Irreversible Inhibition of the *Mycobacterium tuberculosis* β-Lactamase by Clavulanate†

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Received July 27, 2007; Revised Manuscript Received September 12, 2007

ABSTRACT: Members of the β-lactam class of antibiotics, which inhibit the bacterial D,D-transpeptidases involved in cell wall biosynthesis, have never been used systematically in the treatment of *Mycobacterium tuberculosis* infections because of this organism's resistance to β-lactams. The critical resistance factor is the constitutive production of a chromosomally encoded, Ambler class A β-lactamase, BlaC in *M. tuberculosis*. We show that BlaC is an extended spectrum β-lactamase (ESBL) with high levels of penicillinase and cephalosporinase activity as well as measurable activity with carbapenems, including imipenem and meropenem. We have characterized the enzyme's inhibition by three FDA-approved β-lactamase inhibitors: sulbactam, tazobactam, and clavulanate. Sulbactam inhibits the enzyme competitively and reversibly with respect to nitrocefin. Tazobactam inhibits the enzyme in a time-dependent manner, but the activity of the enzyme reappears due to the slow hydrolysis of the covalently acylated enzyme. In contrast, clavulanate reacts with the enzyme quickly to form hydrolytically stable, inactive forms of the enzyme that have been characterized by mass spectrometry. Clavulanate has potential to be used in combination with approved β-lactam antibiotics to treat multi-drug resistant (MDR) and extremely drug resistant (XDR) strains of *M. tuberculosis*.

The emergence of multi-drug resistant and extremely drug resistant (*1*) strains of *Mycobacterium tuberculosis* makes searching for drugs that are effective against these strains imperative. The β-lactam class of antibiotics is one of the most important structural classes of antibacterial compounds, and its members act by inhibiting the bacterial D,D-transpeptidases that are responsible for the final step of peptidoglycan cross-linking (*2*). The major resistance mechanism in bacteria to β-lactams is the production of β-lactamases that catalyze the hydrolysis of the β-lactam ring, preventing their interaction with the D,D-transpeptidases. Among the four classes of β-lactamases (*3*), members of class A are clinically the most prevalent (*4*). They share the same fold and conserved active site residues with the D,D-transpeptidase module of penicillin binding proteins (PBPs), and it is accepted that β-lactamases have evolved from them (*5*). The catalytic mechanism (Figure 1) involves activation of the nucleophilic, catalytic Ser70, by Glu166 and Lys73 (*6*), formation of a covalent acyl−enzyme complex, and finally hydrolysis of the ester bond between the enzyme and the ring-opened product by a water molecule (*7*). β-Lactams have never been used systematically in the treatment of tuberculosis, since an early report in 1949 showed that *M. tuberculosis* contains an active penicillinase (*8*) and subsequent studies demonstrating that the organism is resistant to these antibiotics (*9*). Modern chemotherapy of tuberculosis instead relies on the 6 month co-administration of four compounds: isoniazid, rifampicin, ethambutol, and pyrazinamide. However, β-lactams, alone or in combination with β-lactamase inhibitors, have been shown to be effective in vitro, and in mice and humans infected with *M. tuberculosis* (*10*−*14*). The low permeability of mycobacterial cell wall toward hydrophilic molecules and the nature of the mycobacterial peptidoglycan were thought to be additional causes of β-lactam resistance in *M. tuberculosis* (*15*, *16*). However, Chambers et al. (*10*) measured permeability coefficients for a number of cephalosporins and penicillins and showed these compounds to be rapidly transported through the outer cell wall of *M. tuberculosis* H37Ra, at rates comparable to those observed for *Pseudomonas aeruginosa*.

More recent studies have shown that the intrinsic β-lactam resistance of *M. tuberculosis* is primarily due to the production of an Ambler class A β-lactamase encoded by the *blaC* gene. When *blaC* is deleted, the strain becomes significantly more susceptible (16−32-fold) to penicillins as well as third-generation cephalosporins and carbapenems (*9*). BlaC was initially characterized with regard to its substrate−activity profile 10 years ago (*17*), and its three-dimensional crystal structure was reported last year (*18*). However, its inhibition by FDA-approved β-lactamase inhibitors and its spectrum of substrate specificity have not been thoroughly examined. We describe here the activity of BlaC, which is an unusual chromosomally encoded, extended spectrum β-lactamase, which hydrolyzes penicillins, cephalosporins, and, surprisingly, carbapenems. A detailed analysis of sulbactam, tazobactam, and clavulanate using a combination of kinetic studies and mass spectrometry was used to establish their mechanism of inhibition. The results suggest a therapeutic strategy for the treatment of drug resistant tuberculosis.

METHODS

Production and Purification of BlaC. The *blaC* gene was amplified from genomic DNA and cloned into pET28 using

† This work was supported by NIH Grant AI33696 (to J.S.B.).

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10.1021/bi701506h CCC: $37.00
Published on Web 10/04/2007

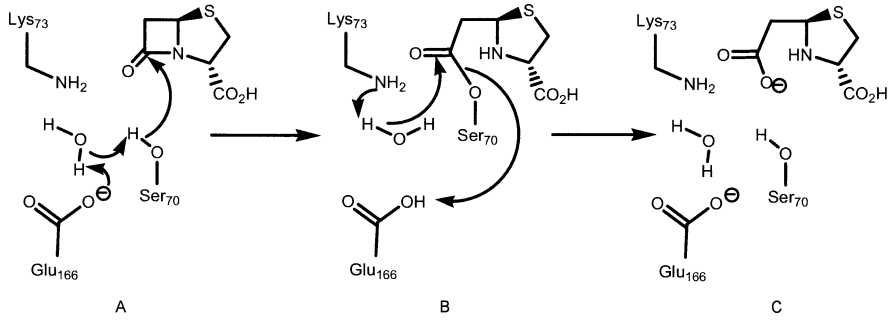

FIGURE 1: Catalytic mechanism of class A β-lactamases. Dual participation of Lys73 and Glu166 for activation of Ser70 (A), formation of the covalent acyl−enzyme intermediate (B), and attack of the ester bond by the activated water molecule (C).

NdeI and HindIII. BlaC was expressed as an N-terminally truncated form, lacking the first 40 amino acids, as previously described (*18*). The plasmid was sequenced and transformed into BL21(DE3) and cultured in LB broth at 37 °C. Induction was performed by the addition of IPTG at 16 °C for 12 h. Cells were harvested, resuspended in 25 mM Tris-HCl, containing 300 mM NaCl (pH 7.5), and disrupted by sonication. After centrifugation, the soluble extract was loaded onto a Ni-NTA agarose column (Qiagen) and eluted with 200 mM imidazole in 25 mM Tris-HCl, containing 300 mM NaCl (pH 7.5). The eluted fractions were dialyzed against 25 mM Tris-HCl, containing 300 mM NaCl (pH 7.5), to remove the imidazole, and thrombin was added to cleave the His_6 N-terminal tag. Size exclusion chromatography was performed using a Superdex 200 Hi-Load 26-60 column (Amersham Pharmacia Biotech) using 25 mM Tris-HCl, containing 300 mM NaCl (pH 7.5), as a buffer.

Kinetics. The steady state rate of hydrolysis of the β-lactam ring was monitored as a decrease in the absorbance in the UV region, as previously described (*19*). Assays using the chromogenic cephalosporins, CENTA (a gift from R. Pratt, Wesleyan University, Middletown, CT) and nitrocefin (Beckton Dickinson), were performed at 405 nm (ϵ = 6400 M^{-1} cm^{-1}) and 486 nm (ϵ = 20 500 M^{-1} cm^{-1}), respectively. Assays were performed in 100 mM MES (pH 6.4). Reactions were initiated by the addition of enzyme at a concentration varying from 1.7 nM to 0.3 μM, depending on the substrate used. Initial velocity kinetic data were fit to

$$v = V[S]/(K + [S]) \quad (1)$$

where v is the initial velocity, V is the maximal velocity, and K is the Michaelis constant for the substrate, S.

Inhibition Studies. Clavulanate and tazobactam (Sigma) and sulbactam (LKT Laboratories) were used at concentrations ranging from 0 to 70 μM, using ca. 0.2 nM BlaC and 100 μM nitrocefin in 100 mM MES (pH 6.4). Time courses were followed for 10 min. For slow-onset inhibition, reaction velocities as a function of time were fitted to eq 2

$$[P] = v_s t + \frac{v_1 - v_s}{k_{iso}}[1 - \exp(-k_{iso} t)] \quad (2)$$

where [P] is the concentration of the product, v_i and v_s are the initial and final velocities, respectively, of the reaction in the presence of inhibitor, k_{iso} is the apparent first-order rate constant for the interconversion between v_i and v_s, and t is time.

We have used the classical model for such inhibition, which includes the rapid binding of I to the enzyme to form the EI complex, followed by a slower isomerization of the complex to form the EI* complex.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

Using this model, the rate constants that describe k_{iso} are given by eq 3, where K_i equals k_{-1}/k_1.

$$k_{iso} = k_{-2} + \frac{k_2[I]}{K_i + [I]} \quad (3)$$

For tazobactam, exhibiting a non-zero intercept value for k_{iso} versus [tazobactam], K_i* was calculated using eq 4:

$$K_i^* = K_i \frac{k_{-2}}{k_2 + k_{-2}} \quad (4)$$

For reversible competitive inhibition, velocities were fitted to the equation

$$v = \frac{V_{max}[S]}{[S] + K_m\left(1 + \frac{[I]}{K_i}\right)} \quad (5)$$

The analysis of the recovery of β-lactamase activity was performed by incubating the enzyme (20 μM) with the three β-lactamase inhibitors (100 μM) in 100 mM MES (pH 6.4). Initial velocities were measured at different times using a final concentration of 0.8 nM enzyme in 100 mM MES (pH 6.4) and 200 μM nitrocefin.

Mass Spectrometry. All mass spectra were acquired on a 9.4 T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Ionspec, Lake Forest, CA). To avoid salt interference, BlaC was dialyzed and concentrated in 10 mM MES (pH 6.4) to a concentration of 200 μM. The stock solution was diluted to 20 μM, and inhibitors were added at a final concentration of 100 μM; 10 pmol of enzyme was directly injected onto a C18 column at a rate of 10 μL/min in 50% acetonitrile containing 0.1% TFA. The molecular mass of each protein sample was determined for the 25+ charge state using the equation $m = (m/z \times 25) - 25$ on the isotopic centroid.

RESULTS

Production and Purification of BlaC. BlaC was expressed in *Escherichia coli* cells and purified in two steps, essentially

Table 1: Kinetic Parameters of BlaC with β-Lactams

substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})
ampicillin	8 ± 0.3	660 ± 20	$(8.1 \pm 0.4) \times 10^7$
amoxicillin	22 ± 1	340 ± 20	$(1.5 \pm 0.1) \times 10^7$
penicillin G	19 ± 0.9	560 ± 20	$(2.9 \pm 0.2) \times 10^7$
penicillin V	69 ± 8	2100 ± 270	$(3.1 \pm 0.5) \times 10^7$
piperacillin	59 ± 5	690 ± 50	$(1.2 \pm 0.1) \times 10^7$
cephalosporin C	114 ± 7	1070 ± 30	$(9.4 \pm 0.6) \times 10^6$
cephalotin	152 ± 25	490 ± 100	$(3.2 \pm 0.8) \times 10^6$
cefuroxime	5100 ± 1200	490 ± 150	$(9.6 \pm 3.7) \times 10^4$
cefamandole	184 ± 15	3500 ± 300	$(1.9 \pm 0.2) \times 10^7$
cefoxitin	127 ± 16	48 ± 6	$(3.8 \pm 0.7) \times 10^5$
ceftazidime	280 ± 40	2.0 ± 0.3	$(7.4 \pm 1.7) \times 10^3$
ceftriaxone	520 ± 14	49 ± 17	$(9.3 \pm 4.1) \times 10^4$
cefotaxime	5570 ± 1360	380 ± 120	$(6.9 \pm 2.8) \times 10^4$
nitrocefin	57 ± 2	6680 ± 260	$(1.17 \pm 0.06) \times 10^8$
CENTA	195 ± 19	1770 ± 190	$(9.1 \pm 1.3) \times 10^6$
imipenem	9.4 ± 0.1	10 ± 1	$(9.2 \pm 0.9) \times 10^5$
meropenem	3.4 ± 0.7	0.08 ± 0.01	$(2.3 \pm 0.5) \times 10^4$

as previously described (*18*). Size exclusion chromatography shows that BlaC elutes at a position expected for the monomer in solution, and the purity was >95% according to Coomassie staining. The overall yield was 125 mg/L of culture.

Substrate Specificity. Initial rate kinetics were used to determined the steady state kinetic parameters for a wide variety of substrates at pH 6.4 (Table 1). BlaC exhibits extraordinarily broad substrate specificity, with high k_{cat} values for both penicillins and cephalosporins. For penicillins, especially ampicillin, the k_{cat}/K_m values are close to 10^8 M^{-1} min^{-1}, values that suggest that the enzyme operates with these substrates at the diffusion-limited rate. The k_{cat}/K_m values observed for the cephalosporins range from ca. 10^7 to 10^4 M^{-1} min^{-1}. As the enzyme can also hydrolyze second- and third-generation cephalosporins (cefuroxime, cefamandole, cefoxitin, ceftazidime, ceftriaxone, and cefotaxime), this enzyme is a naturally occurring, chromosomally encoded extended spectrum β-lactamase (ESBL). In general, ESBLs are derivatives of β-lactamases that have acquired mutations after antibiotic exposure (*20*), which is clearly not the case for the *M. tuberculosis* BlaC or the β-lactamase from *Mycobacterium fortuitum* described recently (*21*). Perhaps most surprisingly, BlaC is capable of hydrolyzing both imipenem and, to a lesser extent, meropenem. Usually the carbapenems, such as meropenem, are potent inhibitors of Ambler class A β-lactamases. It has been suggested that the 6(7)α position occupied by bulky groups in penem β-lactams interacts with a conserved asparagine residue (Asn132) of the SDN motif of penicillin binding proteins and β-lactamases (*22*). This residue is a glycine residue in BlaC, and this substitution is likely to be responsible for the carbapenemase activity, as it is unique among class A β-lactamases (*23*).

Inhibition by β-Lactamase Inhibitors. We investigated the inhibition patterns of the FDA-approved β-lactamase inhibitors clavulanate, tazobactam, and sulbactam. Sulbactam

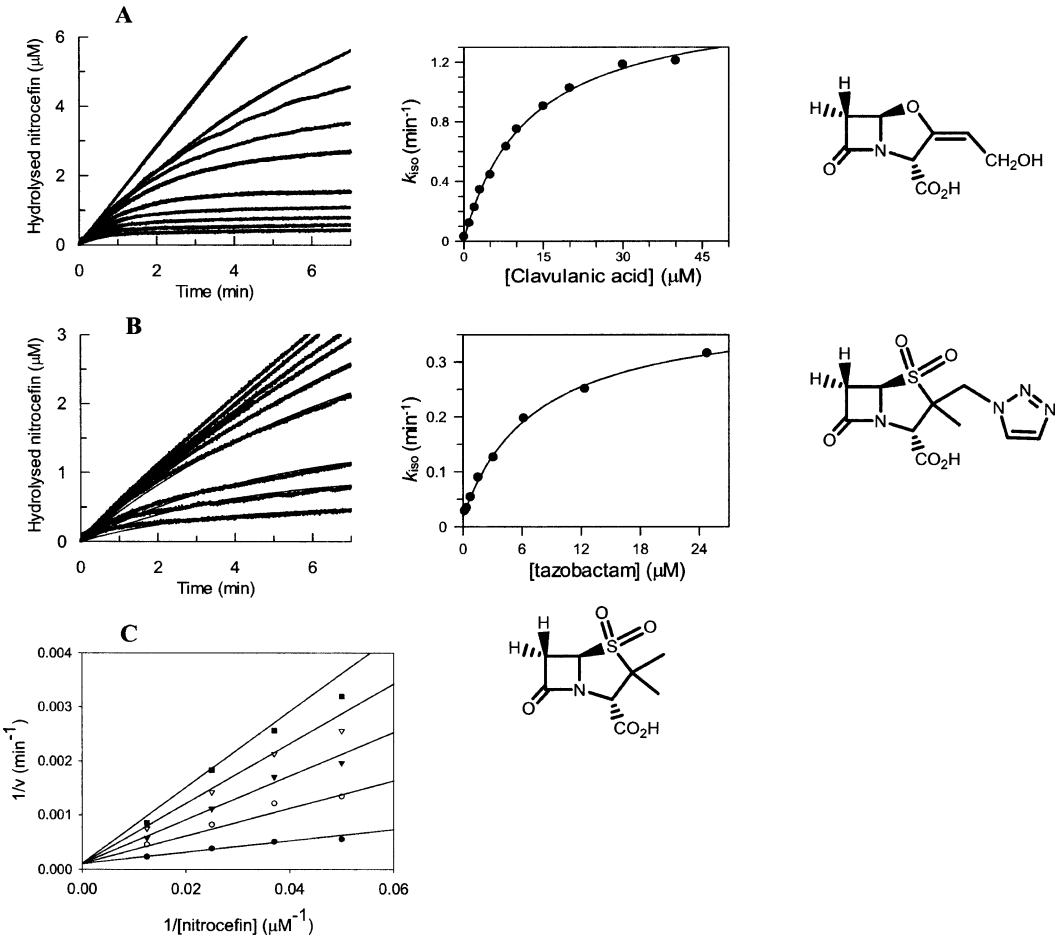

FIGURE 2: Inhibition profiles of *M. tuberculosis* BlaC. Clavulanate (A), tazobactam (B), and sulbactam (C) structures are shown at the right. Times courses are shown in the left panels. After being fit to eq 2, values of k_{iso} are plotted vs inhibitor concentration in the right panels. For sulbactam, initial velocities were determined at sulbactam concentrations of 4 (■), 3 (▽), 2 (▼), 1 (○), and 0 μM (●).

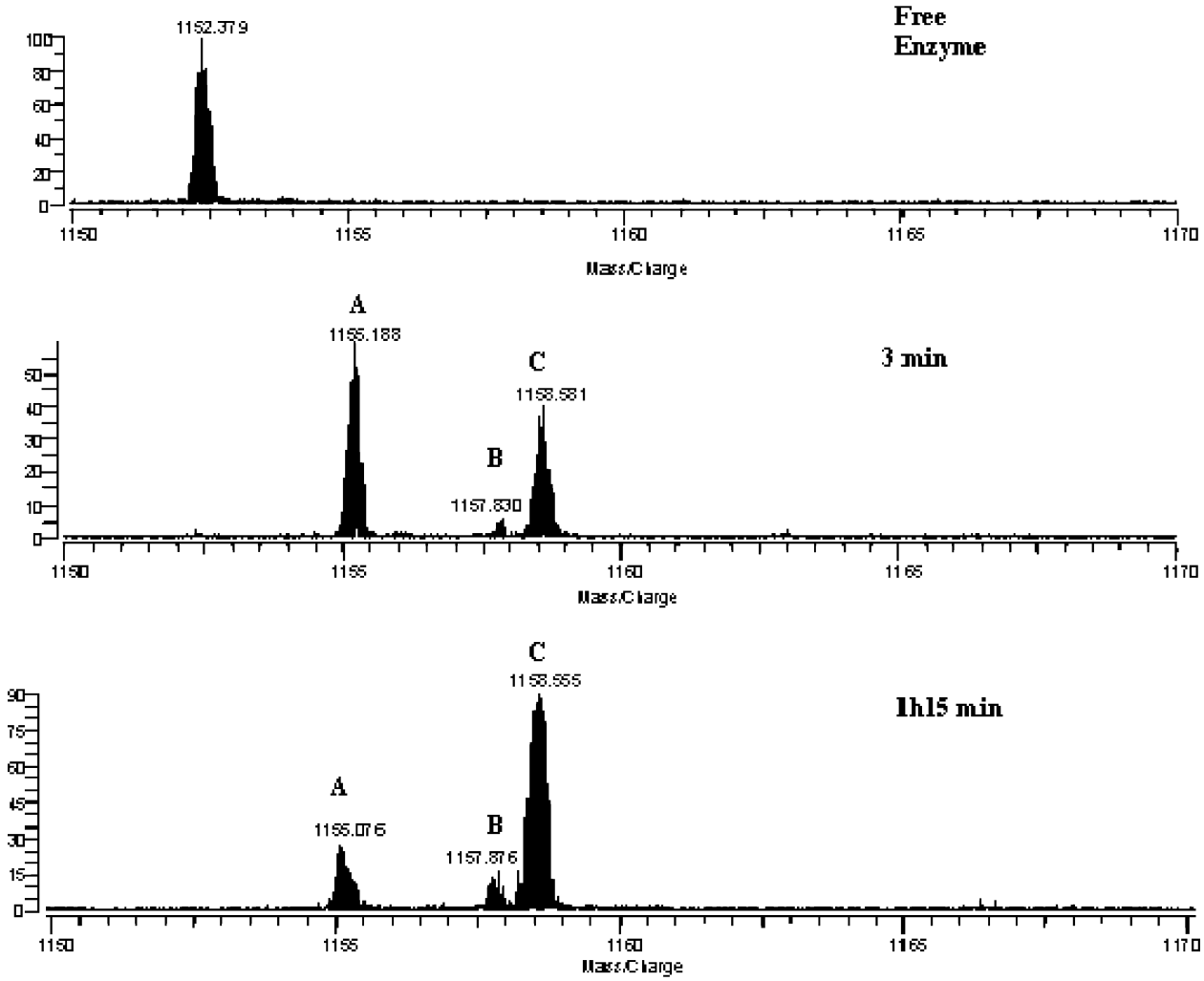

FIGURE 3: FTICR mass spectra of BlaC acyl adducts with clavulanate. Only the 25+ charge states are shown. Free enzyme is shown in the top panel, with an experimental weight of 28 784.475. Three minutes after the addition of clavulanate (middle panel), three enzyme forms are formed: peak A, 28 854.70, $\Delta m = +70.22$; peak B, 28 920.75, $\Delta m = +136.27$; and peak C, 28 939.52, $\Delta m = +154.40$. The bottom panel shows that all three peaks remain after incubation for 1 h.

displayed classical, linear competitive inhibition versus nitrocefin, inhibiting the enzyme with a K_i value of 0.70 ± 0.05 μM. Both tazobactam and clavulanate inhibited BlaC in a time-dependent manner, reminiscent of slow-onset inhibition (Figure 2). When time courses are fit to eq 2 and the determined values of k_{iso} are plotted against tazobactam concentration (Figure 2B), a non-zero intercept is observed. This requires that the EI ↔ EI* isomerization be reversible and allows the values of K_i and K_i* to be calculated to be 7.5 ± 0.8 and 0.34 ± 0.12 μM, respectively. The values of k_2 and k_{-2} were determined from the value of k_{iso} at zero and infinite tazobactam concentrations. These values were determined to be 0.38 ± 0.01 min^{-1} (k_2) and 0.018 ± 0.004 min^{-1} (k_{-2}) for tazobactam. In contrast, using clavulanate, the plot of k_{iso} versus clavulanate concentration reveals an intercept value that cannot be distinguished from zero (Figure 2A). This suggests that once the E−clavulanate* complex is formed, it cannot go back to the E−clavulanate complex, making the step irreversible. The K_i value for clavulanate is 12.1 μM, and the k_2 is 1.6 ± 0.04 min^{-1}. The K_i value determined here is slightly higher than the one reported by Wang et al. (*18*), who reported the inhibition to be biphasic.

To identify the acyl−enzyme forms of the sulbactam-, tazobactam-, and clavulanate-inhibited enzyme species (i.e., EI and EI*), we used Fourier transform ion cyclotron resonance (FTICR) mass spectrometry to follow the covalent modification of the enzyme at times after the addition of the inhibitors. The calculated molecular weight of the free enzyme is 27 784.481, and the experimental molecular weight is 28 784.475. Using tazobactam, the mass spectrum reveals that the enzyme is covalently modified within 5 min and exists in two major forms: one with the entire tazobactam molecule bound (Enz + 300.95, predicted, +300.29) and a second with an increment of mass of 69.95 (Figure S1 of the Supporting Information). This latter complex is presumably the propionaldehyde ester (+70.06) that has been previously observed after enzyme acylation and inhibitor fragmentation with penicillin sulfones (*24*). However, within 12 min of mixing BlaC with tazobactam, peaks in the mass spectrum corresponding to the free enzyme appear, and after 45 min, the acylated forms have completely disappeared. Full activity was recovered after 45 min, suggesting that the enzyme was slowly hydrolyzing the acylated enzyme serine residue (S70) (Figure S1). Similarly for sulbactam, we found that covalent adducts could be observed immediately upon mixing with BlaC and, as with tazobactam, two peaks corresponding to the mass of the E−inhibitor complex (Enz + 233.40, predicted +233.24) as well as the aldehyde (Enz

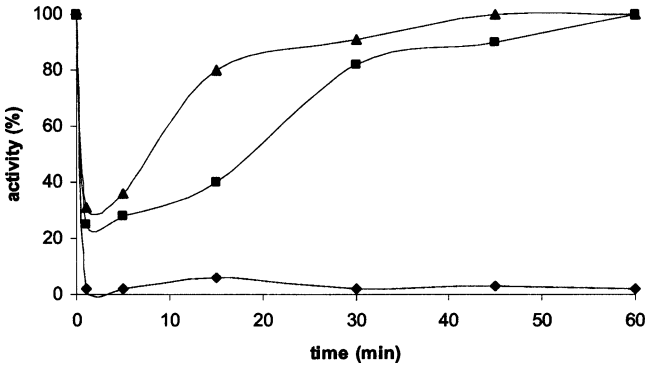

FIGURE 4: Recovery of BlaC activity after incubation with β-lactamase inhibitors. Enzyme (20 μM) was incubated with 100 μM sulbactam (▲), 100 μM tazobactam (■), or 100 μM clavulanate (●), and activity was determined at the indicated times.

+ 69.57). However, these complexes were rapidly hydrolyzed, and within 30 min, the mass spectrum revealed only the native, unmodified enzyme form (Figure S2 of the Supporting Information), and full activity was recovered (Figure 4). The fact that sulbactam does not exhibit slow-onset inhibition likely reflects the very rapid isomerization rate with sulbactam compared to that with tazobactam.

Within 3 min of the addition of clavulanate, three peaks are observed in the mass spectrum, none of which is the intact BlaC−clavulanate covalent adduct (Figure 3, predicted E−clavulanate, 27 784.481 + 199.16). The lowest-molecular weight adduct is the enzyme−propionaldehyde form (Figure 3, peak A; Enz + 70.22) observed with tazobactam and sulbactam. The other two forms appear at Enz + 136.27 (Figure 3, peak B) and Enz + 154.40 (Figure 3, peak C). One of these other two forms corresponds to the enzyme adduct of clavulanate after decarboxylation (Enz + 154.40) to generate an imine (or eneamine tautomer) form of inhibitor (*25*−*27*). The other minor component (peak B) is most likely a covalent adduct in which the imine undergoes the additional loss of a water molecule (Enz + 136.12). These latter forms are likely to be stable to hydrolysis, since no BlaC activity can be detected after incubation with clavulanate for 60 min (Figure 4), or even after 12 h (data not shown). The small amount of propionaldehyde adduct is likely to be slowly hydrolyzed by BlaC, since this same adduct is generated by all three β-lactamase inhibitors. It is likely to be continuously generated until all of the enzyme is present in the nonhydrolyzable complexes represented by peaks B and C. Alternatively, clavulanate might uniquely lead to the covalent modification of S130, which has been proposed for clavulanate inhibition of other β-lactamases (*28*).

We propose a mechanism for the inhibition of BlaC by clavulanate based on these mass spectrometry results (Figure 5). Once the catalytic serine residue reacts with the β-lactam ring of clavulanate, causing the rupture of the β-lactam bond, the remaining ring is ruptured to generate the acyclic imine form of the enzyme-bound complex. This intermediate is rapidly decarboxylated due to the presence of the ketone adjacent to the carboxyl group that can stabilize the carbanion generated by decarboxylation. This is the form of the adduct that predominates (ca. 70%) after prolonged incubation of BlaC with clavulanate (Figure 5, compound C). While we show this in Figure 5 as the *trans*-C_3-N_4 imine tautomer, the C_2-C_3-eneamine and N_4-C_5-imine tautomers can presumably also occur, and these would reduce the propensity

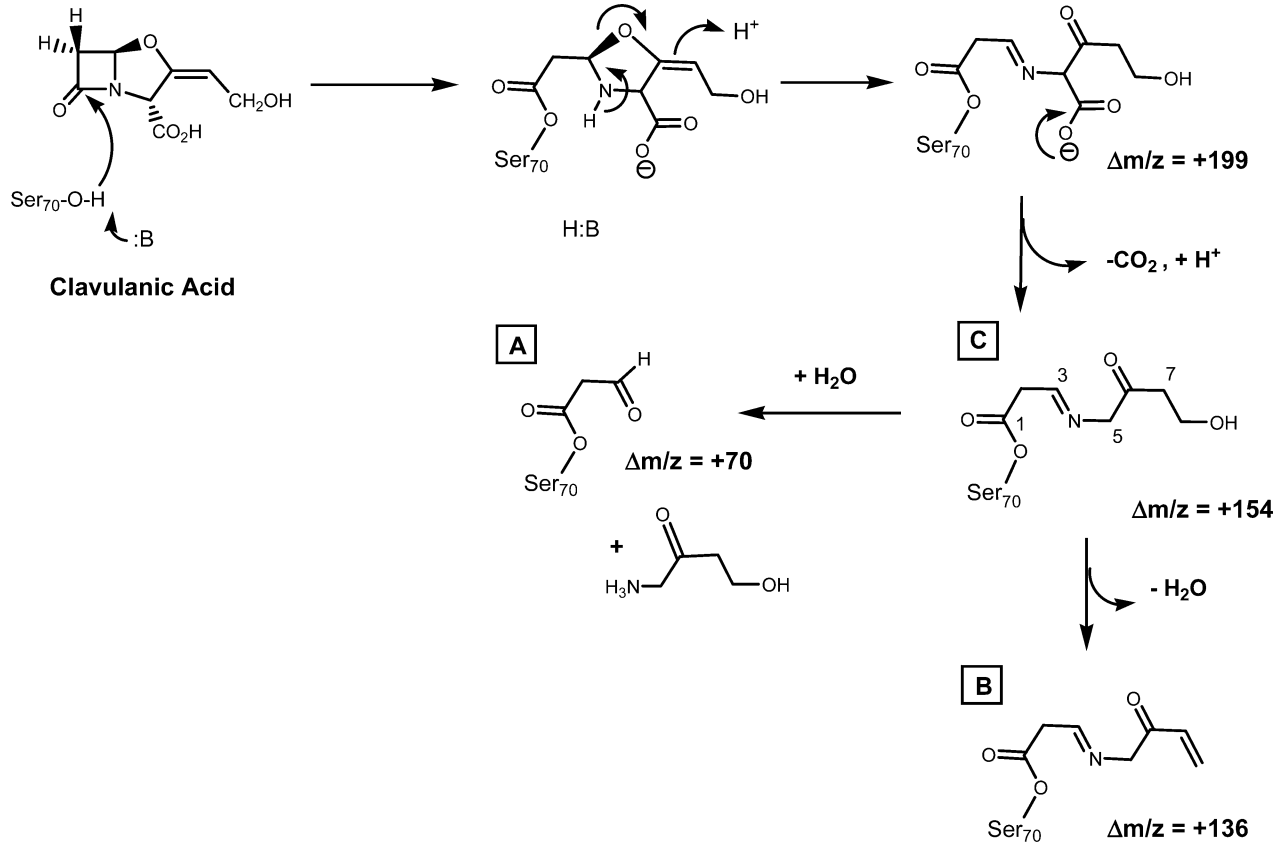

FIGURE 5: Proposed mechanism of BlaC inhibition by clavulanate. After acylation of the clavulanate molecule, rearrangement and decarboxylation lead to the imine (C), which undergoes loss of a water molecule (B) or hydrolysis to form the propionaldehyde (A).

for hydrolysis. Small amounts of a covalent adduct formed by the dehydration of compound C to generate compound B are observed, and this dehydration chemistry has not previously been reported. The imine of compound C can be hydrolyzed to generate the propionaldehyde ester that is also the product of the tazobactam and sulbactam reactions (Figure 5, compound A). This propionaldehyde covalent adduct is presumably unstable and is hydrolyzed since it is also formed transiently with tazobactam and sulbactam. Only clavulanate leads to the stable inactivation of the enzyme, and we therefore propose that the inactive forms of the enzyme are compounds C and B in Figure 5.

DISCUSSION

Tazobactam and sulbactam, members of the penicillin sulfone family of β-lactamase inhibitors, inhibit BlaC, but the covalent adduct generated is unstable and is hydrolyzed; on the other hand, clavulanate leads to the stable inactivation of the enzyme. Since 1983, there have been episodic reports of the in vitro and in vivo efficacy of combination β-lactam/β-lactamase inhibitor therapy for *M. tuberculosis* infections. One of the first of these showed that the combination of amoxicillin and clavulanate was bactericidal in vitro against 14 of 15 *M. tuberculosis* isolates that were tested (*13*). All penicillins, cephalosporins, and imipenem tested have very high affinity for the *M. tuberculosis* H37Ra penicillin binding proteins, and although they are inactive on their own, the MIC value of amoxicillin can be reduced from >16 to 0.5 μg/mL by the co-administration with clavulanate (*10*). The combination of amoxicillin and clavulanate has also been used clinically in the treatment of tuberculosis patients, including those harboring multi-drug resistant strains, and shown to have early bactericidal activity comparable to that of other drugs used to treat tuberculosis with the exception of isoniazid (*11*, *14*). The results presented here clarify the kinetic and mechanistic differences between the various β-lactamase inhibitors and reveal that clavulanate is unique among the β-lactamase inhibitors in being able to stably inhibit BlaC. A combination of clavulanate and a β-lactam, perhaps imipenem which has recently been shown to have activity on its own in the treatment of human and murine tuberculosis (*12*), should be explored in the treatment of currently untreatable multi-drug and extremely drug resistant (*1*) *M. tuberculosis* infections.

SUPPORTING INFORMATION AVAILABLE

Mass spectra at various times of BlaC complexes of tazobactam (Figure S1) and sulbactam (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI701506H